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RESEARCH ARTICLE

Therapeutic effects of different durations of acupuncture on rats with middle cerebral artery occlusion

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Abstract

Acupuncture is regarded as an effective therapy for cerebral ischemia. Different acupuncture manipulations and durations may result in different therapeutic effects. In the present study, the *Neiguan* (PC6) acupoint of rats with occluded middle cerebral arteries was needled at a fixed frequency (3 Hz) with different durations, *i.e.*, 5, 60 and 180 seconds under a twisting-rotating acupuncture method. Results showed that different durations of acupuncture had different therapeutic effects, with 60 seconds yielding a better therapeutic effect than the other two groups. This duration of treatment demonstrated rapid cerebral blood flow, encouraging recovery of neurological function, and small cerebral infarct volume. Experimental findings indicated that under 3 Hz frequency, the treatment of needling *Neiguan* for 60 seconds is effective for ischemic stroke.

***Key Words:** nerve regeneration; middle cerebral artery occlusion; manipulation; Neiguan; cerebral infarction volume; twisting-rotating method; duration; frequency; cerebral blood flow; neural regeneration*

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Introduction

Acupuncture therapy has been proven as an effective means in treating cerebral ischemia. Previous studies have indicated that acupuncture exerts therapeutic effects in promoting cerebral blood flow (Qian et al., 2009; Du et al., 2011; Kim et al., 2013), preventing apoptosis of nerve cells (Zhou et al., 2011a; Wang et al., 2012), improving neurobehavioral performance (Chang et al., 2012; Wang et al., 2012) and regulating protein and gene expression (Guo et al., 2004; Lin and Hsieh, 2010; Pan et al., 2012). According to the theory of acupuncture and moxibustion in traditional Chinese medicine, the *Neiguan* (PC6) acupoint is the collateral acupoint of the Pericardium Meridian of Hand *Jueyin*, and the Pericardium Meridian is the main vessel of the heart that governs blood circulation in the whole body (Liu et al., 2013). Therefore, acupuncture at the *Neiguan* acupoint has the effect of regulating blood circulation in the brain. Modern research has also shown that acupuncture at the *Neiguan* acupoint could produce specific therapeutic effects in the treatment of ischemic stroke (Fan et al., 2009; Zhou et al., 2011b). According to evidence-based research, the *Neiguan* acupoint was found to be one of the ten most frequently used acupoints in the treatment of ischemic stroke (Li, 2009).

In both laboratory and clinical studies, acupuncture manipulation has been overlooked compared with other factors, such as the selection of acupoints or acupuncture methods. The variation of acupuncture manipulations by different acupuncture operators in these studies makes it difficult to achieve uniform standards for acupuncture manipulation. Whether the impact of acupuncture manipulation on therapeutic efficacy is significant or not is a question of great concern for acupuncture researchers. As a physical stimulus, acupuncture creates a particular level of stimulation and acupuncture manipulation is the approach to regulate the amount of stimulation. Acupuncture manipulation is an extensive concept that includes duration, frequency, angle, depth and other aspects. Duration, the operating time of acupuncture, is one of the most commonly used factors in the regulation of acupuncture manipulation, so the present study selected duration as a representative factor to explore the impact of acupuncture manipulation on the efficacy of acupuncture. In previous studies, needling *Neiguan* using the thrusting-lifting method to treat cerebral ischemia showed

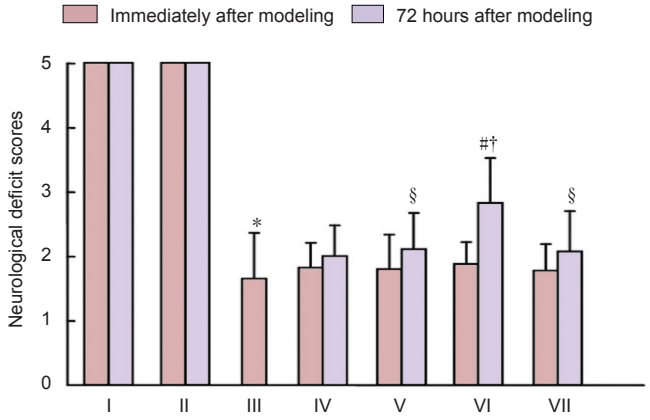

Figure 1 Effects of different durations of acupuncture on neurological deficit scores in rats with middle cerebral artery occlusion.
Results are expressed as the mean ± SD of 18 rats in each group. One-way analysis of variance was used to compare differences between groups, and the least significant difference test was used to compare between two groups. ∗$P < 0.05$, *vs.* normal group; #$P < 0.05$, *vs.* model group; †$P < 0.05$, *vs.* non-acupuncture group; §$P < 0.05$, *vs.* 60 seconds acupuncture group. I: Normal group; II: sham group; III: model group; IV: non-acupuncture group; V: 5 seconds acupuncture group; VI: 60 seconds acupuncture group; VII: 180 seconds acupuncture group.

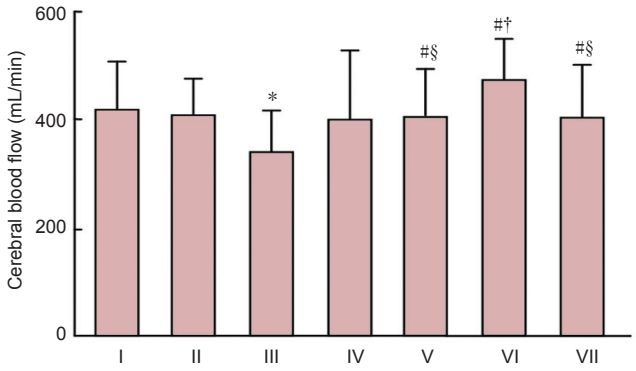

Figure 2 Effects of different durations of acupuncture on cerebral blood flow in rats with middle cerebral artery occlusion.
Results are expressed as the mean ± SD of 18 rats in each group. One-way analysis of variance was used to compare differences between groups, and the least significant difference test was used to compare between two groups. ∗$P < 0.05$, *vs.* normal group; #$P < 0.05$, *vs.* model group; †$P < 0.05$, *vs.* non-acupuncture group; §$P < 0.05$, *vs.* 60 seconds acupuncture group. I: Normal group; II: sham group; III: model group; IV: non-acupuncture group; V: 5 seconds acupuncture group; VI: 60 seconds acupuncture group; VII: 180 seconds acupuncture group.

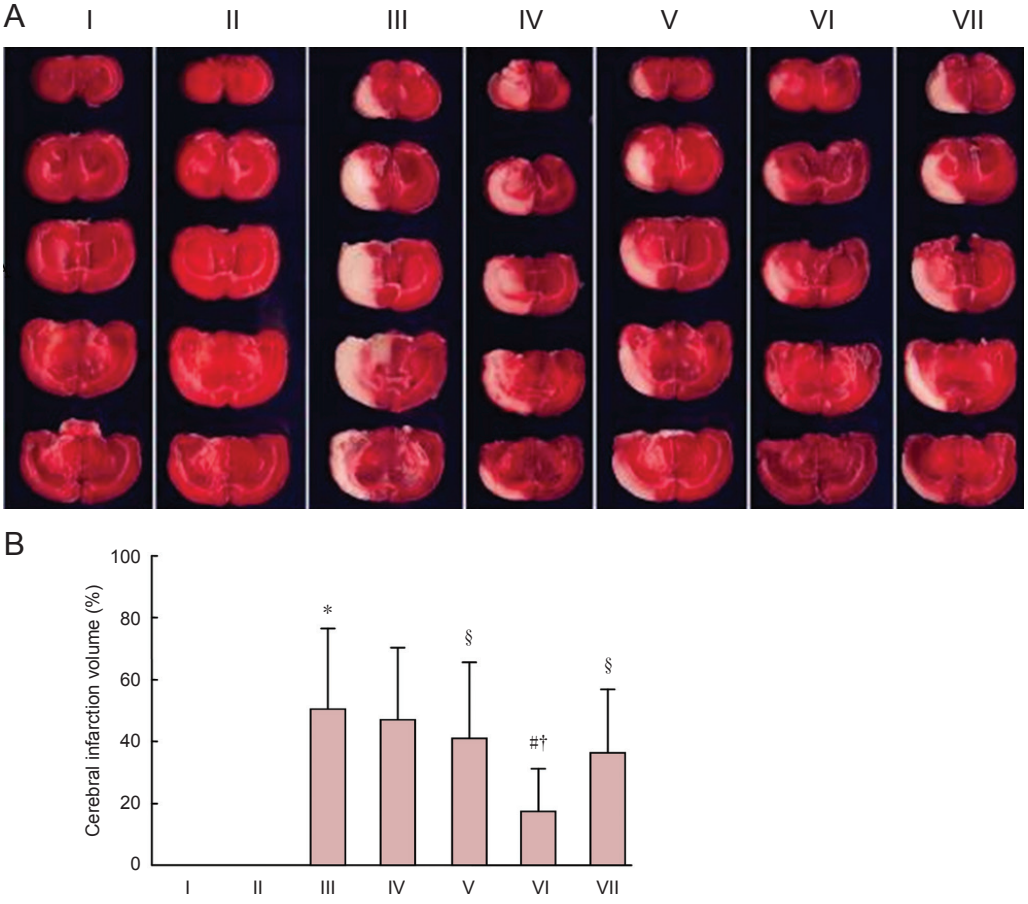

Figure 3 Effects of different durations of acupuncture on cerebral infarction volume in middle cerebral artery occlusion rats.
(A) Cerebral infarction volume in middle cerebral artery occlusion rats following 2,3,5-triphenyltetrazolium chloride staining. The white area is infarct region and red is normal tissue. (B) Cerebral infarction volume ratio of middle cerebral artery occlusion rats. Results are expressed as the mean ± SD of 18 rats in each group. One-way analysis of variance was used to compare differences between groups, and the least significant difference test was used to compare between two groups. ∗$P < 0.05$, *vs.* normal group; #$P < 0.05$, *vs.* model group; †$P < 0.05$, *vs.* non-acupuncture group; §$P < 0.05$, *vs.* 60 seconds acupuncture group. I: Normal group; II: sham group; III: model group; IV: non-acupuncture group; V: 5 seconds acupuncture group; VI: 60 seconds acupuncture group; VII: 180 seconds acupuncture group.

affirmative therapeutic outcomes, with different parameters of duration and frequencies varying in therapeutic effects (Zhang et al., 2013b). To further prove the significance of acupuncture manipulation to acupuncture effects, the present study adopted the twisting-rotating method to treat a rat model of middle cerebral artery occlusion by needling the *Neiguan* acupoint, in a broader attempt to determine the preferable duration of needling *Neiguan* to treat ischemic stroke.

Materials and Methods

Animals

Male, specific pathogen-free Wistar rats, aged 3 months and weighing 250–300 g were provided from the Laboratory Animal Center of People's Liberation Army Academy of Military Medical Sciences, Beijing, China (license No. SCXK (Army) 2007-004). Animals were acclimated to the animal quarters for at least 3 days before experiments, and were allowed standard laboratory diet and water *ad libitum*. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and the experimental protocol was approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine in China.

A total of 126 rats were randomly and equally assigned to seven groups: control, sham, model, non-acupuncture, and 5, 60 or 180 seconds of acupuncture.

Establishing middle cerebral artery occlusion models

A modified Zea-longa's thread ligation method (Longa et al., 1989) was applied to duplicate the middle cerebral artery occlusion model. Briefly, rats in the model, non-acupuncture, 5 seconds acupuncture, 60 seconds acupuncture and 180 seconds acupuncture groups were fasted for 12 hours with free access to water and anesthetized by intraperitoneal injection of 10% hydration chloral hydrate (350 mg/kg). Rats were then fixed in the dorsal position on the surgery board, neck skin and muscle were incised, and the common carotid artery, the external carotid artery and the internal carotid artery on the left were isolated. The external carotid artery and the proximal end (near the heart) of the common carotid were ligated with No. 0 suture line. A small hole was pierced with a 1 mL syringe needle at the proximal end of the common carotid. A 0.28 mm nylon thread was inserted from the hole into the internal carotid until resistance was met, with an intracranial depth of 18–20 mm. Blood flow in the left middle cerebral artery was blocked by the nylon thread. The nylon thread was then ligated with the common carotid artery and muscle and skin were sutured. The nylon thread was not applied in rats of the sham group. Once the animals recovered, they were returned to their cages with food and water available *ad libitum*. This intraluminal suture model of the middle cerebral artery occlusion produces reliable and permanent focal cerebral ischemia.

Acupuncture intervention

In the 5, 60, and 180 seconds acupuncture groups, the *Neiguan* acupoint on the right side was needled with different durations, *i.e.*, 5, 60 and 180 seconds, under the same twisting-rotating frequency (3 Hz). In the normal, sham and non-acupuncture groups, rats did not receive any acupuncture intervention, but were also handled six times with the acupuncture groups in the experimental period. According to the Acupoint Location of Commonly-used Experimental Animals in Experimental Acupuncture Science (Li, 2003), the *Neiguan* acupoint was located at the forefoot, between the ulna and the radius, about 3 mm from the joint. Sterile disposable stainless steel needles (length: 40 mm, diameter: 0.30 mm; Hwatuo, Suzhou Medical Supplies Factory Co., Ltd., Suzhou, China) were used in this study. All acupuncture manipulation was performed by a twisting-rotating controlled acupuncture machine (Haifu Technology Co., Ltd., Chongqing, China). The *Neiguan* acupoint was perpendicularly needled to a depth of 2 mm. Rats were needled for the first time after they regained consciousness from anesthesia (normally 3–5 hours after middle cerebral artery occlusion surgery) and received five more acupuncture treatments in the subsequent 72 hours.

In the model group, rats did not receive any acupuncture intervention. Rats in this group underwent neurobehavioral testing and cerebral blood flow detection as soon as they recovered from anesthesia. They were then decapitated to allow for cerebral infarction volume measurement. Observations were performed immediately after modeling in the model group, and at 72 hours after modeling in the non-acupuncture group.

Evaluation of neurological function

Deficits in neurological function were assessed according to the Zausinger's 6-point method (Zausinger et al., 2000) after middle cerebral artery occlusion rats recovered from anesthesia. The standards used to obtain the Zausinger six-point score were as follows: 0, the rat could not spontaneously walk; 1, the rat rotated towards the side opposite to the lesion with free walking; 2, the rat rotated towards the side opposite to the lesion when its tail was seized; 3, the resistance to the lateral pressure was decreased in the side opposite to the lesion; 4, the rat could not unbend front paws or entire forelimb on the side opposite to the lesion; 5, the rat had no neurological function defect. Rats with a score of 1–3 were considered to have undergone successful cerebral ischemia injury and rats with a score of 0, 4 or 5 were not used for further experimentation. All rats were assessed at 72 hours after modeling.

Detection of cerebral blood flow

Cerebral blood flow detection was performed at 72 hours after modeling in all groups but the model group. Rats were fixed on a self-made stereotaxic instrument after anesthesia. A midline incision was made on the scalp to expose the anterior fontanel. A small bone window 1 mm posterior to the anterior fontanel and 3 mm left of the midline was produced with a dental drill. The measurement probe was placed on the left cerebral hemisphere. Cerebral blood flow was continuously measured by laser Doppler flowmetry (DRT4, Moor

Instrument, Wilmington, DE, USA) for 1 minute for each rat.

Measurement of cerebral infarction volume by 2,3,5-triphenyltetrazolium chloride (TTC) staining

After neurobehavioral tests and cerebral blood flow detection were completed, all rats were euthanized with chloral hydrate and brains were collected quickly and placed at a temperature of −20°C for 30 minutes. The brain tissue was cut into five coronal sections at 3 mm thick and stained with a 2% solution of TTC in PBS at a temperature of 37°C for 20 minutes, followed by 4% paraformaldehyde buffer for fixation. The stained sections were photographed, and the digital images were analyzed by a computer-assisted image system with Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). The total infarction volume was calculated as the sum of the area of the brain infarction multiplied by the thickness of each section (3 mm). The infarction volume was presented as a percentage of the total ipsilateral hemispheric volume, which can be calculated by using the following equation: [(the volume of the intact contralateral hemisphere) – (the volume of the intact ipsilateral hemisphere – the volume of infarcted tissue in the ipsilateral hemisphere)]/the volume of the intact contralateral hemisphere) × 100%. This rectified measurement equation corrects for edema in the total infarct volume.

Statistical analysis

All data were expressed as the mean ± SD. Statistical analyses were performed with SPSS 16.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used to compare differences between groups, and the least significant difference test was used to compare between two groups. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of different durations of acupuncture on neurological function in middle cerebral artery occlusion rats

Neurological deficit scores were significantly decreased in the model group ($P < 0.05$) when compared with normal and sham groups. Neurological deficit scores in rats from the model group showed no significant difference between the 5 seconds acupuncture group, 180 seconds acupuncture group and non-acupuncture group ($P > 0.05$). Compared with the non-acupuncture group, neurological deficit scores in rats from the 60 seconds acupuncture group were significantly increased ($P < 0.05$). Compared with the 5 seconds acupuncture group and 180 seconds acupuncture group, neurological deficit scores in the rats from the 60 seconds acupuncture group were also significantly increased ($P < 0.05$). No significant difference was seen in neurological deficit scores in rats from the 5 seconds acupuncture group and 180 seconds acupuncture group ($P > 0.05$; **Figure 1**).

Effects of different durations of acupuncture on cerebral blood flow in rats with middle cerebral artery occlusion

Compared with normal and sham groups, cerebral blood flow was significantly decreased in the model group ($P < 0.05$). Cerebral blood flow in rats from the model and non-acupuncture groups was similar ($P > 0.05$). Compared with the model group, cerebral blood flow in the rats from all three acupuncture groups was significantly increased ($P < 0.05$). Compared with the 5 seconds acupuncture group and 180 seconds acupuncture group, cerebral blood flow in the rats of the 60 seconds acupuncture group was also significantly increased ($P < 0.05$). Cerebral blood flow in rats from the 5 seconds acupuncture group and 180 seconds acupuncture group showed no significant difference ($P > 0.05$; **Figure 2**).

Effects of different durations of acupuncture on cerebral infarction volume of middle cerebral artery occlusion rats

Cerebral infarction was clearly seen in the model group ($P < 0.05$) and infarction volume was reduced in all three acupuncture groups ($P < 0.05$), while no infarction was observed in the normal and sham groups. Cerebral infarction volume in rats from the model group, non-acupuncture group, 5 seconds acupuncture group and 180 seconds acupuncture group were similar ($P > 0.05$). Compared with the non-acupuncture group, cerebral infarction volume in rats from the 60 seconds acupuncture group was significantly decreased ($P < 0.05$). Compared with the 5 seconds acupuncture group and 180 seconds acupuncture group, cerebral infarction volume in rats from the 60 seconds acupuncture group was also significantly decreased ($P < 0.05$). Cerebral infarction volume in rats from the 5 seconds acupuncture group and 180 seconds acupuncture group showed no significant difference ($P > 0.05$; **Figure 3**).

Discussion

The middle cerebral artery occlusion animal model has been proven to be a classic model of human cerebral ischemia (Zhang and Ben, 2012; Sun et al., 2013; Zhang et al., 2013a; Du et al., 2014; Mei and Zhang, 2014). In our study, the middle cerebral artery occlusion rat model was successfully induced using the previously described Zea-longa's thread ligation method (Longa et al., 1989) and evaluated later by neurobehavioral scores. Apparent ischemic areas in the brain samples were also shown using TTC staining methods. The model group showed significant differences in neurobehavioral scores, cerebral blood flow and cerebral infarction volume compared with normal and sham groups. No difference was seen between normal and sham groups.

The therapeutic effect of acupuncture on stroke is still controversial, and relevant meta-analysis shows that acupuncture therapy does not bring extra effects in addition to physical training or placebo (Sze et al., 2002; Hopwood et al., 2008; Kong et al., 2010), while other studies show acupuncture can produce certain beneficial effects in the treatment of stroke (Wu et al., 2010; Zhang et al., 2014). Considering the ability to spontaneously recover after stroke (Cramer, 2008; Colombo et al., 2013), we specifically added a non-acupuncture group in this study. Results showed that even though the non-acupuncture group revealed a trend towards recov-

ery, no significant difference was found when compared with the model group. Meanwhile, the 60 seconds acupuncture group had a significant improvement in neurological deficit scores, cerebral blood flow and infarction volume compared with the non-acupuncture, 5 seconds, and 180 seconds acupuncture groups. Our results proved that the body's ability for spontaneous recovery is very limited and acupuncture is effective in treating ischemic stroke.

Despite the variation of acupuncture manipulation in laboratory and clinical studies, the effect of acupuncture therapy cannot be separated from acupuncture manipulation. Since lifting-thrusting and twisting-rotating are the two most common methods of acupuncture manipulation, our study selected the twisting-rotating method and a distinct difference in therapeutic effect was seen between different durations of acupuncture. Cerebral blood flow increased in all three acupuncture groups compared with the model group and still a significant difference can be seen between the 60 seconds acupuncture group and the other two acupuncture groups. Research has found that the growth in the blood supply to the cerebral ischemic area can effectively provide an important source of neurotrophic support to newly generated neurons and also serves as a route for neuroblasts to move into the recovering peri-infarct region (Tsai et al., 2006; Goldman and Chen, 2011). So the improvement of cerebral blood flow is closely related to neural injury symptoms after ischemic stroke. Since 3 Hz, 60 seconds acupuncture showed the highest increase in cerebral blood flow, its protective effect on neurological function may be the best after ischemic stroke.

The therapeutic effect seen in the 60 seconds acupuncture group was not seen in either the 5 seconds or 180 seconds groups at the same frequency. Only the 60 seconds acupuncture group showed a significant improvement in neurological function and cerebral infarction volume compared with the model and non-acupuncture groups. We assume that there is a threshold level of acupuncture stimulation and therapeutic effect can only be achieved above this threshold level. But once acupuncture stimulation achieves the threshold level, it should be maintained at this point. Acupuncture tolerance has been shown to be a common phenomenon when acupuncture stimulation is too strong, which can bring side effects of acupuncture treatment (Han et al., 1981). In our study, 60 seconds is an appropriate duration of acupuncture with a fixed frequency of 3 Hz, which coincides with Shi's acupuncture manipulation quantitative theory which states that 60–180 seconds is the most appropriate length of acupuncture duration (Bian and Zhang, 2003).

In one of our former studies (Chang et al., 2014), we adopted the lifting-thrusting method to needle *Neiguan* acupoint to treat middle cerebral artery occlusion rats, and a duration of 5 seconds and 60 seconds showed the best results, while 180 seconds showed valid results at the frequency of 3 Hz. Given the incongruence between this past study and the present findings, the acupuncture manipulation method may also be an important factor contributing to acupuncture effect and the dose-effect relationships of acupuncture under different manipulation methods may differ from each other. However, both our present and former studies have illustrated the importance of acupuncture manipulation to acupuncture effect. Only appropriate acupuncture manipulation leads to fine therapeutic effect. This partly explained the negative results in some previous studies of acupuncture. Results obtained from studies that ignored the importance of acupuncture manipulation may not be accurate.

Since acupuncture manipulation has such an important impact on the effect of acupuncture, it is necessary for it to be given a special focus in acupuncture research. However, the variation in acupuncture manipulations of different acupuncturists makes it difficult to form a consistent standard, which restricts the further development of acupuncture therapy, presenting a major challenge for acupuncture standardization. In our study, we tentatively used a twisting-rotating controlled acupuncture machine to perform acupuncture manipulation. Although using acupuncture manipulation instruments to replace manual acupuncture is still in the experimental stages, it guarantees the consistency of acupuncture manipulation and enhances the repeatability of acupuncture research.

In conclusion, acupuncture is an effective therapy in treating ischemic stroke and manipulation is a vital influential factor on acupuncture effect. In the present study, 60 seconds is the optimal duration in acupuncturing the *Neiguan* acupoint for ischemic stroke under fixed frequency *i.e.*, 3 Hz. However, acupuncture manipulation is quite a broad term which not only refers to acupuncture duration but also includes acupuncture frequency, amplitude and angle. In the present study, a focus was placed on acupuncture duration. Whether other factors influence the therapeutic effect of acupuncture still needs further exploration. In our study, the therapeutic effects of acupuncture were observed within 72 hours, but the effects of acupuncture on the later progression of cerebral ischemia are still unknown. The mechanisms underlying how acupuncture manipulation influences the therapeutic effect of acupuncture remain unclear, and it is these underlying biological mechanisms of acupuncture manipulation that will form the basis of ongoing research into the effects of acupuncture in treating ischemic stroke.

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Author contributions: *ZHM and XNF conceived the study and prepared the initial protocol. CZ drafted the manuscript and participated in the study design. YW and XYZ participated in completing the study. GT and SZD helped to analyze the data. All authors approved the final version of the manuscript.*

Conflicts of interest: *None declared.*

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